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TAXONOMIC STUDY OF SHALLOW-WATER OCTOPUSES (CEPHALOPODA: OCTOPODIDAE) IN JAPAN AND ADJACENT WATERS USING MITOCHONDRIAL GENES WITH PERSPECTIVES ON OCTOPUS DNA BARCODING

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ABSTRACT

A recent study revised the generic level taxonomy of the family Octopodidae, and several species groups that were previously included in *Octopus* are now recognized as independent genera. However, knowledge regarding phylogenetic relationships among taxa in the family Octopodidae is still poor. The phylogenetic relationships of shallow-water benthic octopus species in Japan and adjacent waters were examined in the present study using mitochondrial DNA cytochrome *c* oxidase subunits I and III (COI and COIII, respectively) sequence data, and the taxonomic statuses of the octopus species were compared to those derived by morphological identification. We obtained COI (657 bp) and COIII (464 bp) sequences from 45 taxa assigned to 34 species of seven genera. Samples used were voucher specimens stored in the National Museum of Nature and Science, Tokyo. Sequences of the genes of both *Haliphron atlanticus* and *Tremoctopus violaceus* from GenBank were included as outgroup taxa. Additionally, sequences of the genes of *Octopus vulgaris* obtained from Mediterranean coast of France, which is type locality of this species, was also included from GenBank in order to discuss about status of Japanese *O. vulgaris*. Through phylogenetic analyses, we recognized *Callistoctopus* and a distinct group including four *Octopus* species and an *Enteroctopus* species, which was designated the "*Enteroctopus* group" in the present study, with high bootstrap support. *Amphioctopus* was not completely distinguished because it sometimes formed a clade together with *Hapalochlaena*, which shows morphological affinities with *Amphioctopus*. *Abdopus* + *O. laqueus* + *O. cyanea* and *O. vulgaris* + *O. oliveri* were recognized as distinct groups, but the phylogenetic support for these groups was weak. For identifying species using short DNA sequences, sequence divergences were calculated using COI sequences. Sequences within and among species were generally divergent enough to discriminate the species, but several overlaps with intra- and interspecific divergence caused by poor taxonomic resolution were also observed. Although performing species identification using DNA sequences from shallow-water benthic octopus species is premature, the results in this study indicated the possible application of COI sequences in octopus species identification, thereby providing a preliminary dataset for future DNA barcoding of octopus.

Key words: phylogenetic relationships, shallow-water benthic octopus species, mitochondrial genes, species identification, DNA barcoding.

INTRODUCTION

Octopus is a cosmopolitan genus and has been a source of taxonomic confusion with respect to the family Octopodidae. A recent study demonstrated that the generic-level taxonomy of the family Octopodidae needs major revision, and that several species groups that were previously included in *Octopus* are now recognizable as independent genera (Norman & Hochberg, 2005). Several new or resurrected genera were presented based on those species groups, and the generic statuses of several species were changed. However, phylogenetic support for these newly independent genera is still poor, and *Octopus* still remains a heterogeneous assemblage as has been demonstrated in recent years through several studies.

Several studies have been conducted to determine phylogenetic relationships among octopus species based on molecular analysis. The non-monophyletic status of *Octopus* was

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demonstrated in previous studies based on molecular analysis using mitochondrial genes (Carlini et al., 2001; Takumiya et al., 2005). Moreover, molecular analysis data obtained using two mitochondrial DNA genes, cytochrome *c* oxidase subunit III (CO III) and cytochrome b (CYTB), and a nuclear DNA elongation factor gene indicated the non-monophyletic status of *Octopus* and established several monophyletic groups, which might represent independent genera (Guzik et al., 2005), thereby supporting recent taxonomic changes. However, knowledge regarding phylogenetic relationships among taxa in the family Octopodidae is still restricted to a few species, and further accumulation of molecular data, especially data associated with morphological information, is required.

Here we examined the molecular phylogeny of shallow-water benthic octopus species distributed in Japan and adjacent waters, which include the northern part of the Indo-West Pacific region. Previous studies on the taxonomy of shallow-water benthic octopus species distributed in Japan and adjacent waters have recognized more than 80 species (Sasaki, 1929; Taki, 1942, 1963, 1964; Okutani et al., 1987). Recent studies have revised these previous works, added several new species, and discovered high species diversity within the benthic octopus species in this area (Kubodera & Lu, 2002; Kaneko & Kubodera, 2005; 2007a, b). We analyzed phylogenetic relationships among these diverse octopus taxa using DNA sequence data obtained from mitochondrial cytochrome *c* oxidase subunit I (COI) and COIII genes. Our analysis compared the classification of DNA data obtained from museum-stored materials with their historical classification based on morphology. We studied the phylogenetic relationships of shallow-water benthic octopus species collected from Japan, Taiwan, and Vietnam, and compared the taxonomic statuses of these species with those derived from morphological identification. The results of this study will also provide a provisional dataset for future DNA barcoding of octopus, which was introduced for the Class Cephalopoda at the Cephalopod International Advisory Council in 2006 (Strugnell & Lindgren, 2007).

MATERIALS AND METHODS

Specimens and DNA Tissue Samples

Specimens examined in this study were collected during surveys conducted from 1998 to 2007 in Japan, Taiwan, and Vietnam (Kubodera & Yamada, 1998; Kubodera, 2001; Kubodera & Yamada, 2001; Kaneko, 2006; Kaneko et al., 2008). Specimens were identified to the lowest possible taxon based on morphological characteristics. Small tissue samples were taken from the mantles or arms of the specimens and stored in 99% ethanol for DNA extraction. The specimens were subsequently fixed in 10% formalin and later stored in 40% isopropanol. All specimens and tissue samples were registered and deposited in the National Museum of Nature and Science, Tokyo (NSMT), Japan.

Selection of Specimens for Analysis

From the NSMT collection, 45 specimens were selected, and their taxonomical statuses were identified morphologically. We used “cf.” for provisionally identified species, and the attribution of genus was generally according to a previous study (Norman & Hochberg, 2005).

Polymerase Chain Reaction Amplification and Sequencing

DNA was extracted using a phenol/chloroform extraction protocol. A partial segment of mitochondrial COI was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994). The primers used for mitochondrial COIII were Ooc3F and Ooc3R (Guzik et al., 2005).

Polymerase chain reaction (PCR) was performed in volumes of 50 μL containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 1.0 unit of Taq DNA polymerase (Takara Ex Taq™; Takara Bio Inc., Otsu, Japan). Amplifications for COI were performed using a Takara PCR Thermal Cycler MP (Takara) under the following conditions: 30 s at 94°C, 30 s at 45°C, and 60 s at 72°C (35 cycles). The samples were incubated at 94°C for 3 min before the cyclic reactions and at 72°C for 5 min after completion. Amplifications for mitochondrial COIII were performed under the following conditions: 30 s at 94°C, 30 s at 50°C, and 60 s at 72°C (40 cycles) with the final extension step performed at 72°C for 5 min.

We placed 5 μL of the reaction product on a 1.5% agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer to check the success of the reaction. The remaining PCR product was purified using ExoSAP-IT (Amersham Biosciences, Piscataway, New Jersey, USA).

PCR products were sequenced using the BigDye Terminator Cycle Sequencing v3.1 Cycle

Sequencing Kit (Applied Biosystems, Foster City, California, USA) on an ABI PRISM™ 3100-Avant (Applied Biosystems). Sequencing reactions were performed in volumes of 20 μL containing 3.0 μL of Terminator Ready Reaction Mix, 2.5 μL of 5X sequencing buffer, and 3.2 pmol of the appropriate sequencing primer. The reactions were performed under the following conditions using the Takara PCR Thermal Cycler MP (Takara): 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C (25 cycles). Samples were incubated at 96°C for 1 min before the cyclic reactions. Products were purified using ethanol/EDTA/sodium acetate precipitation according to the sequencing kit manual. Sequencing for COIII samples was performed by Macrogen Inc. (Seoul, South Korea). Sequences were aligned using ATGC ver. 4.0.10 and Genetix ver. 7.0.8 (Genetix Ltd., New Milton, UK).

Data Analysis

We performed maximum parsimony (MP) and maximum likelihood (ML) analyses to construct the phylogenetic trees. MP analyses for COI and COIII datasets were performed separately using PAUP*4.0b10 (Swofford, 2002). In addition, we performed a combined analysis of COI and COIII data because the incongruence length difference test (ILD test; Farris et al., 1994), which was performed using PAUP*4.0b10 (Swofford, 2002; 1,000 replicates of heuristic searches), suggested no significant phylogenetic difference between the two datasets ($P = 0.783$). MP analysis was performed using heuristic searches with 100 random addition analyses and TBR branch swapping. Robustness of each phylogenetic tree was tested by bootstrapping (1,000 replicates).

Prior to performing ML analysis, we searched the best-fit model of evolution using ModelTest (Posada & Crandall, 1998), and the GTR+I+G model (Rodriguez et al., 1990) was selected as the best-fitting model for COI (invariable sites: 0.5755, gamma distribution parameter: 1.1120), COIII (invariable sites: 0.4655, gamma distribution parameter: 0.7436), and combined datasets (invariable sites: 0.5226, gamma distribution parameter: 0.8136). ML analyses were performed using PhyML (Guindon & Gascuel, 2003; Guindon et al., 2005). Robustness of each phylogenetic tree was tested by bootstrapping (100 replicates) with nearest-neighbor interchange branch swapping. Sequences of the genes of both *Haliphron atlanticus* and *Tremoctopus violaceus* from GenBank were included as outgroup taxa (accession numbers: AF377964, AF377978, GU288525 and GU288522). Additionally, sequences of the genes of *Octopus vulgaris* obtained from Mediterranean coast of France (Allcock et al., 2006), which is type locality of this species, was added to the datasets from GenBank (accession numbers: EF016328, EF016319). *Octopus vulgaris* is a type species of genus *Octopus*, which is a core problem of the recent generic confusion of Octopodidae.

RESULTS

Morphological Taxonomy

The selected 45 specimens were identified as 34 species and were assigned to the following seven genera: *Abdopus*, *Amphioctopus*, *Callistoctopus*, *Cistopus*, *Enteroctopus*, *Hapalochlaena*, and *Octopus*. Specimen data listed in Table 1 included species name, NSMT registered number, collection date and site, and site number as shown in Figure 1, and GenBank accession numbers.

The study also included *Callistoctopus minor*, the taxonomic status of which has been assigned as *Octopus* or as unclear (Toll & Voss, 1998; Norman & Hochberg, 2005). However, we placed this species in *Callistoctopus* because we considered that its morphological characteristics, such as long arms with the dorsal pair being the longest, moderate to high gill lamellae counts (10–14 per demibranch), and a large copulatory organ on the right arm 3 are diagnostic of the genus (Robson, 1929; Taki, 1942, 1964; Voss, 1981; Norman, 1992a). This species has been morphologically classified into the following three subspecies: *O. minor typicus*, *O. minor pardalis*, and *O. minor minor* (Sasaki, 1929; Toll & Voss, 1998). Here we classified the specimen as *O. minor typicus*.

Although *O. megalops* was considered to be a synonym of *O. hongkongensis* (Gleadall, 1993), we found a specimen in our collection having characteristics similar to those of *O. hongkongensis*, but the specimen had an extremely large eye, which is a notable characteristic of *O. megalops* (Taki, 1964). Here we classified this specimen as *O.* cf. *megalops* and included it in the dataset of molecular analysis.

Three unidentified species (*Callistoctopus* sp. 1–3) (Kaneko et al., 2008) were also included in the dataset.

TABLE 1. List of specimens used for analysis in this study. Abbreviations indicate the areas of sample collection (EC, East China Sea; JP, Japan; OG, Ogasawara Islands; RK, Ryukyu Islands; TW, Taiwan; and VN, Vietnam), and site numbers are associated with the numbers in Fig. 1.

Species	Museum No. (NSMT-Mo.)	Collection Date	Collection sites	Site No.	Gen. Bank Acc. No. COI	Gen. Bank Acc. No. COIII
Amphioctopus marginatus	74708	00/06/04	East China Sea	EC	AB430521	AB573195
Amphioctopus marginatus	74816	02/01/14	Nha Trang, Vietnam	VN2	AB385870	AB573196
Amphioctopus marginatus	74597	01/10/19	Uraga, Tokyo Bay, Japan	JP4	AB430522	AB573194
Amphioctopus cf. *neglectus*	74819	02/01/14	Nha Trang, Vietnam	VN2	AB385872	AB573190
Amphioctopus cf. *rex*	75217	03/12/06	Nha Trang, Vietnam	VN2	AB385871	AB573191
Amphioctopus cf. *siamensis*	76109	07/05/26	Wuchi market, Taiwan	TW	AB430516	AB573192
Amphioctopus aegina	76110	07/05/26	Wuchi market, Taiwan	TW	AB430515	AB573189
Amphioctopus kagoshimensis	76116	03/02/01	Jogashima Is., Japan	JP4	AB430520	AB573193
Amphioctopus ovulum	72114	00/05/10	Tosa Bay, Japan	JP6	AB430524	AB573197
Amphioctopus ovulum	74751	00/06/04	East China Sea	EC	AB430523	AB573198
Amphioctopus cf. *ovulum*	74820	02/01/19	Nha Trang, Vietnam	VN2	AB385873	AB573199
Amphioctopus fangsiao	74749	00/06/24	East China Sea	EC	AB430518	AB573186
Amphioctopus fangsiao	71636	99/11/09	Seto Inland Sea, Japan	JP5	AB430519	AB573187
Amphioctopus fangsiao	76107	07/05/26	Wuchi market, Taiwan	TW	AB430517	AB573188
Hapalochlaena fasciata	76117	02/10/05	Tateyama, Chiba, Japan	JP3	AB430529	AB573212
Hapalochlaena lunulata	76017	06/11/20	Miyagi Is. Okinawa, Japan	RK1	AB430530	AB573213
Hapalochlaena maculosa	76111	07/05/26	Wuchi market, Taiwan	TW	AB430531	AB573214
Abdopus aculeatus	75974	03/11/24	Iriomote Is. Okinawa, Japan	RK2	AB430514	AB573185
Abdopus abaculus	76100	07/10/28	Ikei Is. Okinawa, Japan	RK1	AB430513	AB573184
Cistopus cf. *indicus*	74822	02/09/22	Cat Ba, Vietnam	VN1	AB385878	AB573210
Octopus vulgaris	71640	99/10/19	Seto Inland Sea, Japan	JP5	AB430546	AB573217
Octopus vulgaris	74600	00/06/18	Misaki, Sagami Bay, Japan	JP4	AB430547	AB573219
Octopus vulgaris	74775	00/06/19	East China Sea	EC	AB430548	AB573218
Octopus parvus	76119	04/02/20	Misaki, Sagami Bay, Japan	JP4	AB430544	AB573216

(continues)

(continued)

Species	Museum No. (NSMT-Mo.)	Collection Date	Collection sites	Site No.	Gen. Bank Acc. No. COI	Gen. Bank Acc. No. COIII
Octopus cyanea	75911	95/02	Ogasawara Is., Japan	OG	AB430534	AB573223
Octopus cyanea	75995	06/11/30	Nakagusuku, Okinawa, Japan	RK1	AB430535	AB573224
Octopus oliveri	75913	95/02	Ogasawara Is., Japan	OG	AB430532	AB573226
Octopus laqueus	75992	06/11/08	Miyagi Is. Okinawa, Japan	RK1	AB430543	AB573215
Octopus incella	76101	07/11/07	Motobu, Okinawa, Japan	RK1	AB430542	AB573225
Octopus wolfi	76113	07/08/15	Kadena, Okinawa, Japan	RK1	AB430545	AB573227
Octopus megalops	72110	99/11/16	Tosa Bay, Japan	JP6	AB430538	AB573220
Octopus conispadiceus	75134	01/09/15	Off Kushiro, Hokkaido, Japan	JP2	AB430533	AB573222
Octopus longispadiceus	72212	00/10/20	Seto Inland Sea, Japan	JP5	AB430539	AB573228
Octopus hongkongensis	76118	03/03	Misaki, Sagami Bay, Japan	JP4	AB430537	AB573221
Enteroctopus dofleini	76115	03/	Wakkanai, Hokkaido, Japan	JP1	AB430536	AB573211
Callistoctopus luteus	76114	07/11/10	Ohdo, Okinawa, Japan	RK1	AB430526	AB573208
Callistoctopus luteus	74595	00/12/10	Miura, Sagami Bay, Japan	JP4	AB430527	AB573206
Callistoctopus luteus	74814	02/01/14	Nha Trang, Vietnam	VN2	AB385874	AB573207
Callistoctopus aspilosomatis	75986	06/11/08	Miyagi Is. Okinawa, Japan	RK1	AB430525	AB573205
Callistoctopus sp. 1	75219	03/12/06	Nha Trang, Vietnam	VN2	AB385875	AB573203
Callistoctopus sp. 2	75221	03/12/06	Nha Trang, Vietnam	VN2	AB385876	AB573204
Callistoctopus sp. 3	74815	02/01/19	Nha Trang, Vietnam	VN2	AB385877	AB573202
Callistoctopus ornatus	75982	06/11/21	Ohdo, Okinawa, Japan	RK1	AB430528	AB573209
Callistoctopus minor typicus	74731	00/06/18	East China Sea	EC	AB430540	AB573201
Callistoctopus minor typicus	71634	99/11/16	Seto Inland Sea, Japan	JP5	AB430541	AB573200

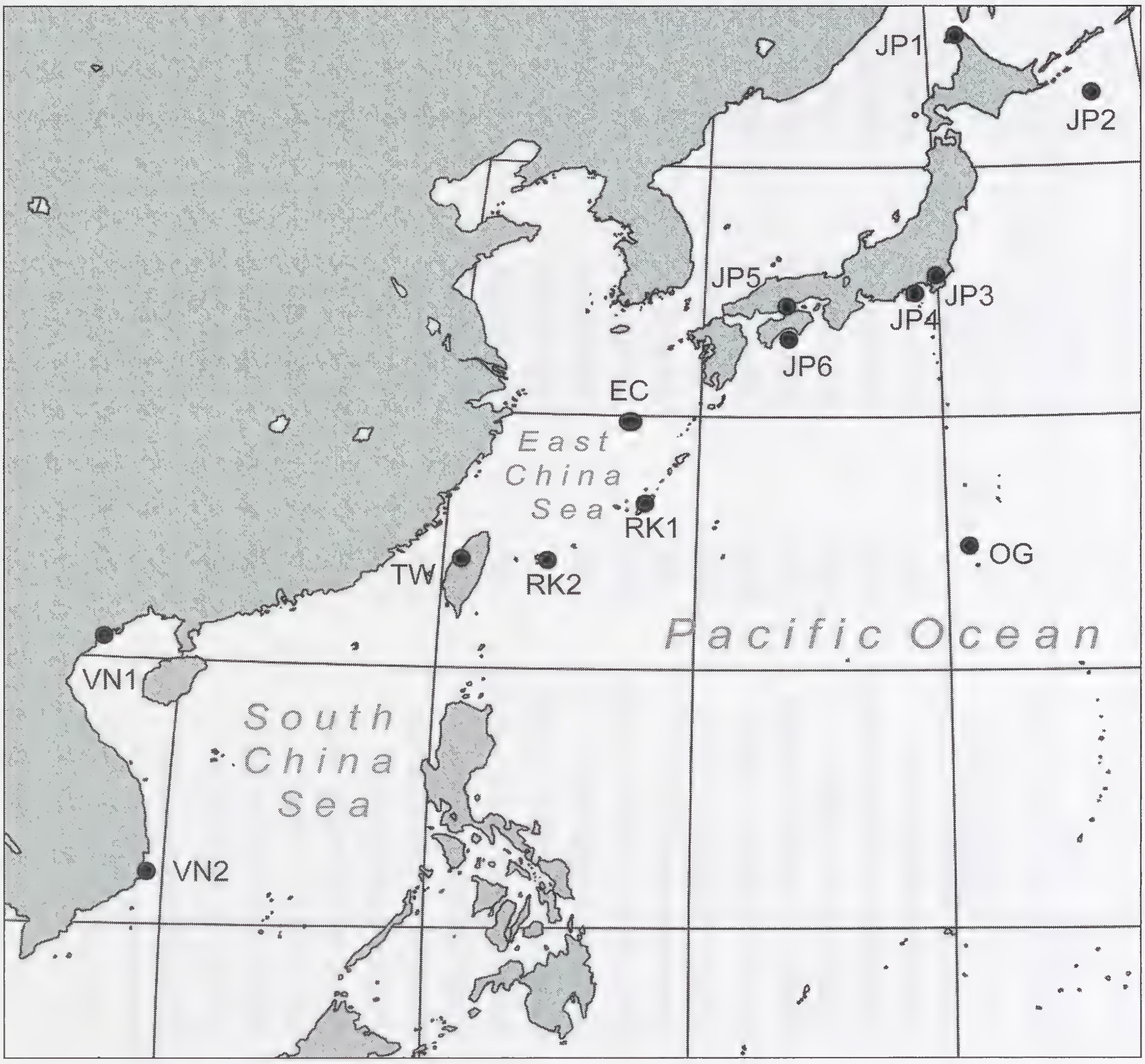

FIG. 1. Specimen collection sites. Abbreviations indicate the areas of sample collection (EC, East China Sea; JP, Japan; OG, Ogasawara Islands; RK, Ryukyu Islands; TW, Taiwan; and VN, Vietnam).

Phylogenetic Analyses

We obtained a total of 1,121 bp from mitochondrial COI (657 bp) and COIII (464 bp) sequences.

The combined ML analysis tree (Fig. 2) supported monophyly of both *Callistoctopus* and *Hapalochlaena*, with a high bootstrap value (100%). A clade including *Enteroctopus dofleini* and 4 *Octopus* species was also supported by a bootstrap value of 100%. In this study, we refer to this clade as the "*Enteroctopus* group." *Amphioctopus* appeared to be monophyletic, but support for monophyly was not high (79%). The two *Abdopus* species were sister taxa with 83% bootstrap support; however, the *Abdopus* + *O. laqueus* + *O. cyanea* clade was supported by a high bootstrap value (94%).

The combined MP analysis tree (Fig. 3) also supported monophyly of *Callistoctopus* and the "*Enteroctopus* group" with high support (> 99%). However, monophyly of *Amphioctopus* was not supported; this genus formed a supported clade together with *Hapalochlaena*. There was low support (74%) for *Abdopus*. In comparison with the ML tree, the MP tree did not find significant support for the *Abdopus* + *O. laqueus* + *O. cyanea* clade (58%).

Both ML and MP trees recovered the *O. vulgaris* + *O. oliveri* clade, but its support was relatively low (≤ 77%) in both topologies. *Octopus vulgaris* from the Mediterranean Sea was included in this clade.

Intra- and Interspecies Sequence Divergence

Seven species of three genera, including 18 specimens, were sampled from multiple localities (Table 1). Sequence divergence in the COI sequence, which was obtained from the same

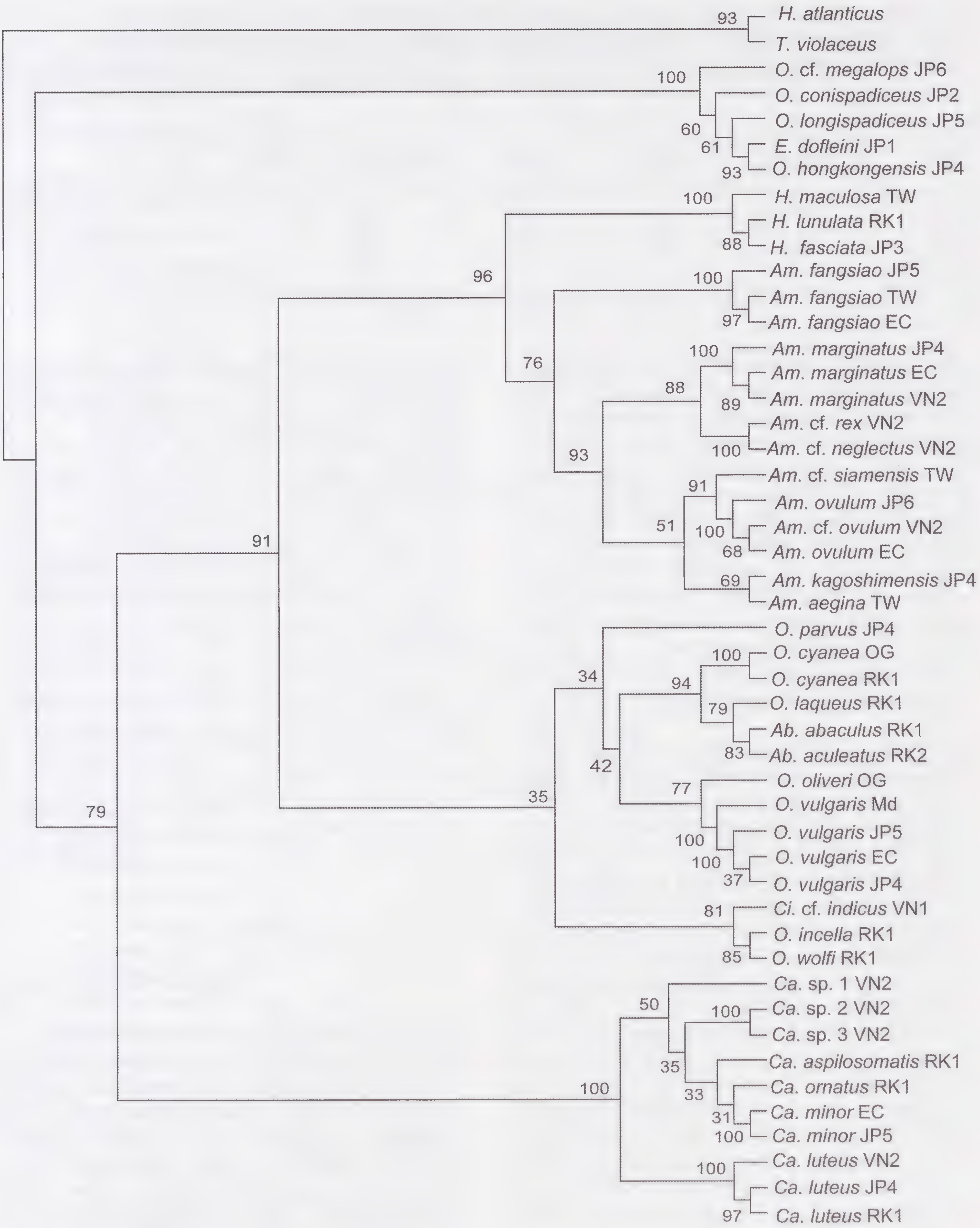

FIG. 2. Phylogram for shallow-water benthic octopus species distributed in Japan and adjacent waters including *Octopus vulgaris* from Mediterranean Sea and for outgroup taxa (*Haliphron atlanticus* and *Tremoctopus violaceus*) based on the maximum likelihood analysis of combined mitochondrial COI and COIII sequences (GTR+G+I model). Numbers beside branches are bootstrap percentages. Abbreviations after the species name indicate the collection site as shown in FIG. 1 (EC, East China Sea; JP, Japan; Md, Mediterranean Sea; OG, Ogasawara Islands; RK, Ryukyu Islands; TW, Taiwan; and VN, Vietnam).

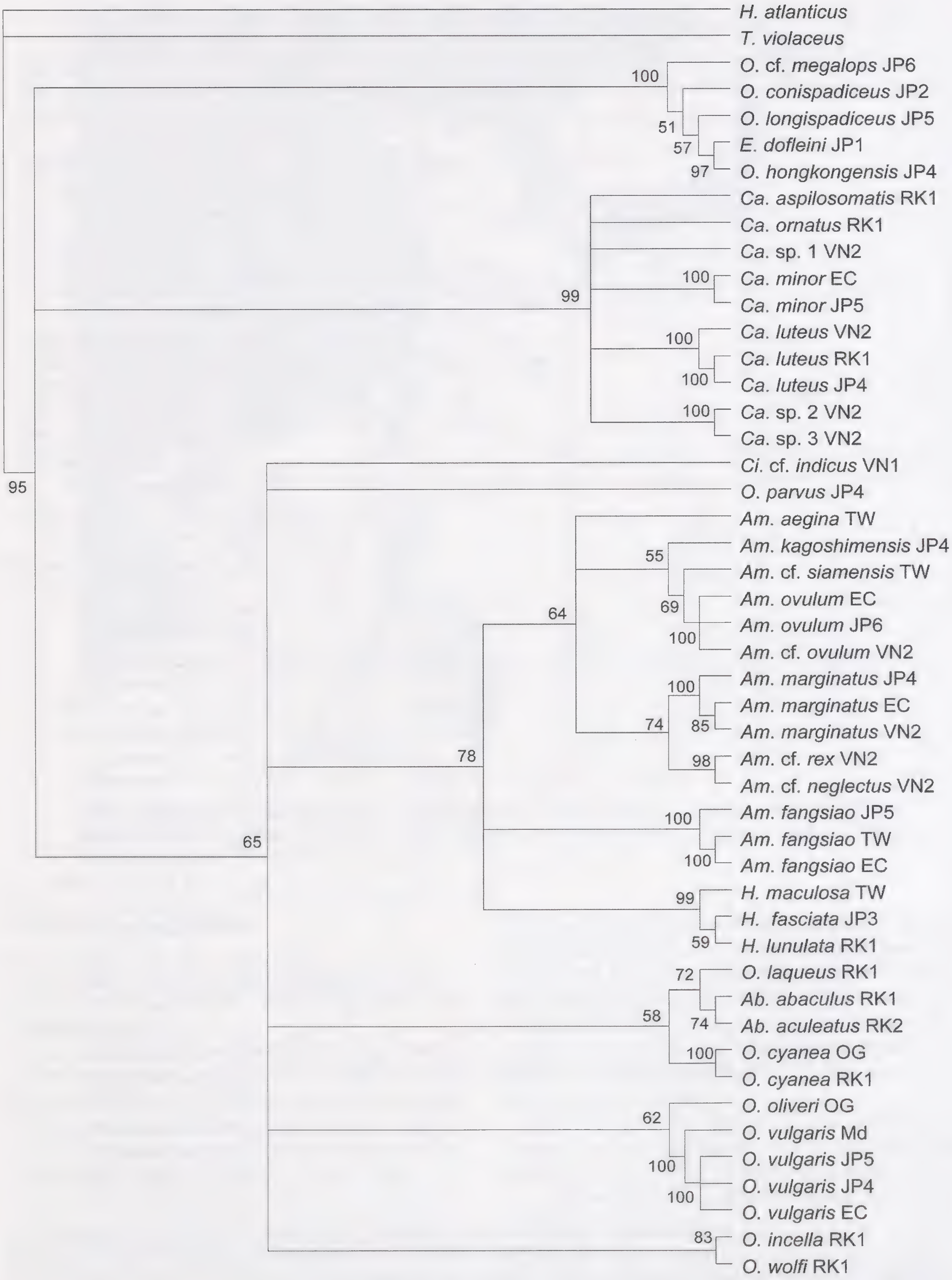

FIG. 3. Phylogram for shallow-water benthic octopus species distributed in Japan and adjacent waters including *Octopus vulgaris* from Mediterranean Sea and for outgroup taxa (*Haliphron atlanticus* and *Tremoctopus violaceus*) based on the maximum parsimony analysis of combined mitochondrial COI and COIII sequences. Numbers beside branches are bootstrap percentages. Abbreviations after the species name indicate the collection site as shown in FIG. 1 (EC, East China Sea; JP, Japan; Md, Mediterranean Sea; OG, Ogasawara Islands; RK, Ryukyu Islands; TW, Taiwan; and VN, Vietnam).

TABLE 2. COI sequence variation within and between sibling species of shallow water benthic octopuses. Pairwise genetic distances were calculated by PAUP* (Swofford, 2002) using the GTR + G + I model of nucleotide evolution. The minimum, max, mean and standard deviation were calculated for pairwise comparisons within species and between sibling species.

	No. of combinations	Min	Max	Mean	SD
Intraspecific	20	0.00	0.07	0.02	0.02
Interspecific (between sibling species)	45	0.04	0.51	0.28	0.11

species from different localities and calculated using the GTR + G + I model, ranged from 0.00 to 0.07, with an overall average of 0.02. On the other hand, divergence among sibling species, which was also calculated using the GTR + G + I model, ranged from 0.04 to 0.51, with an overall average of 0.28 (Table 2). No differences (0.00) were observed between *Am. marginatus* specimens collected from the East China Sea and Vietnam, and *O. vulgaris* specimens collected from the East China Sea and Japan stations 5 and 6. Divergence between Japanese and the Mediterranean *O. vulgaris* was 0.02. The highest intraspecies divergence (0.07) was observed in *Am. fangsiao* specimens collected from Japan station 5, East China Sea, and Taiwan. The lowest interspecies divergence (0.04) was observed in *Ca.* sp. 2 and *Ca.* sp. 3 specimens collected from Vietnam.

DISCUSSION

Taxonomic Implications

Our phylogenetic analyses of shallow-water benthic octopus species distributed in Japan, Taiwan, and Vietnam, using partial mitochondrial gene sequences, recovered clades in support of two less well-understood genera.

A *Callistoctopus* clade received high bootstrap support in both analyses. This genus, previously referred to as the "*Octopus macropus* species group" (Guzik et al., 2005), was not monophyletic in this prior study, falling in a clade with *Grimpella*. Comparing our results with this previous study is difficult because the targeted DNA regions and species in the two studies are quite different. However, our conclusion here is that *Callistoctopus* is a distinct genus, as suggested by Norman & Hochberg (2005).

Monophyly of the "*Enteroctopus* group" in the present study was clearly supported with a high bootstrap value. Of the species therein, only "*dofleini*" has been previously correctly placed in *Enteroctopus*. *Octopus hongkongensis* and *O. conspadiceus* were previously placed in *Paroctopus* (Robson, 1929). However, Norman & Hochberg (2005) included only *P. digueti* in this genus and excluded several species endemic to the North Pacific Ocean. The generic statuses of *megalops* and *longispadiceus* have not yet been determined; currently, they are placed in *Octopus* (Taki, 1964; Takeda, 2003). Our results suggest they should be placed in *Enteroctopus*.

The statuses of *Amphioctopus* and *Hapalochlaena* were equivocal. A *Hapalochlaena* clade was supported by a high bootstrap value in the combined ML tree, but it formed a clade together with *Amphioctopus* in the MP tree. *Hapalochlaena* species are known as blue-ringed octopus and are easily recognized by the dramatic appearance of iridescent blue rings or lines on the dorsal body surface, which distinguishes *Hapalochlaena* as a distinct genus. However, *Hapalochlaena* and *Amphioctopus* share some diagnostic characteristics, such as presence of short arms (2–3× mantle length) and ocelli on the arm base. In addition, *Hapalochlaena* species and *Am. mototi*, a small ocellate *Amphioctopus* species distributed in the Indo-West Pacific, are known to possess strong venom in their salivary gland (Sheumack et al., 1978; Norman, 1992b, 2000). These evidences of morphological affinities and our results of molecular analysis indicate close evolutionary relationships between these two genera.

The affinities of species previously assigned to *Octopus* are still not well understood. We believe that the clade including specimens identified as *O. vulgaris* and *O. oliveri* might truly belong to *Octopus*, because *O. vulgaris* from the Mediterranean Sea, the type species of this genus from the type locality, was included in this clade. Although a number of regional populations of octopus species, which are designated *O. vulgaris*, have been observed and identification of many of these populations needs reexamination, the Japanese population of *O. vulgaris* and Mediterranean population seem to be conspecific in accordance with the low value of sequence divergence among them (0.02). The Mediterranean sample represents genuine *O. vulgaris*, and consequently our result supported the identification of Japanese population.

Abdopus was considered to be a distinct genus, but its bootstrap values were divergent in different topologies (ML tree: 83%; MP tree: 74%). It forms a clade with *O. laqueus* and *O. cyanea* consistently, both of which are distributed in coral reef areas. The topology indicated that the *Abdopus* species and *O. laqueus* showed a close relationship with each other, even though *O. laqueus* never perform arm autotomy, a characteristic behavior of *Abdopus* species. Huffard (2007) reported an *Abdopus* species, *Ab. undulates*, possessing similar morphological characteristics to those of *O. laqueus* collected from Tonga, indicating a close similarity between these taxa. A close relationship was observed between *Abdopus* species and *O. cyanea* (Guzik et al., 2005). This evidence indicates an evolutionary relationship of these species in coral reef areas, regardless of morphological variety. The generic statuses of *O. cyanea* and *O. laqueus*, along with those of a number of recognized and unrecognized coral reef species, await further studies.

Cistopus is characterized by the presence of water pouches on the oral surface of the webs between the arms, and males have a tiny conical ligula without a calamus. This genus inhabits muddy bottom areas of the South China Sea. In the ML tree, *Cistopus* cf. *indicus* formed a clade with *O. incella* and *O. wolfi*, which are pygmy species without water pouches that inhabit clear water coral reef areas. No morphological or ecological affinities were observed among these species, and further investigation is required to resolve their taxonomic status.

Species Identification using COI Region

The use of short DNA sequences for species identification has recently attracted great attention under the term DNA barcoding. The method promises to assign an unknown sample to a known species and to support explorative studies to discover potentially undescribed species. It may also enable the large-scale identification of organisms in ecological or genomic studies (Blaxter, 2004). The sequence divergence at mitochondrial COI regularly enables the discrimination of closely allied species in all animal phyla except Cnidaria (Hebert et al., 2003). Availability of DNA barcoding using COI sequences for cephalopods were demonstrated by Allcock et al. (2011) in their study on deep sea octopus, *Pareledone*. Our study found that the mean value of intraspecific variation at COI in benthic octopus species was much lower than the mean value of interspecific divergence (intraspecific: 0.02; interspecific: 0.28), also indicating that mitochondrial COI would be the appropriate region to discriminate species of the benthic octopus.

However, the accuracy of barcoding for species identification depends on the extent of, and the separation between, intraspecific variation and interspecific divergence in the selected markers. The overlap of genetic variation within species and divergence among sibling species makes barcoding less effective (Meyer & Paulay, 2005). In our result, overlap of intraspecific variation with interspecific divergence also existed. We identified three specimens from different localities as *Am. fangsiao*, but sequence variation among individuals of *Am. fangsiao* was 0.07, which was greater than the minimum value for interspecific divergence (0.04). *Am. fangsiao* is a taxonomically complicated species and is at times referred to as “Iidako.” Some different morphological types have been reported within this species. Two different varieties or subspecies in *Am. fangsiao*, i.e., *Am. fangsiao* var. *typicus* and *Am. fangsiao* var. *echuanus* have been recognized in a previous study (Sasaki, 1929). The diagnostic character of these two types determined by Sasaki is a brick-colored patch on head between eyes, dumbbell or elliptical in shape in var. *typicus*, and crescent shaped in var. *echuanus*. None of our specimens possessed the crescent patch between the eyes. One of the analyzed specimens showed a dumbbell-shaped patch, and the others showed an elliptical patch. The result indicated the presence of cryptic species in *Am. fangsiao*, but at present, this species is treated as a single species comprising a number of synonyms (Gleadall & Naggs, 1991; Norman & Hochberg, 2005). Comprehensive revision of this species together with resolution of associated nomenclatural problems is required. Not only in the case of *Am. fangsiao*, but a number of taxa in Octopoda face the same problem. Several names of these taxa that can cause misunderstanding should be reorganized in accordance with zoological nomenclature standards for identification by both genetic and morphological methods.

Studies on the effectiveness of DNA barcoding on taxonomically well-understood taxa demonstrated that DNA barcoding is much less effective for identification in taxa where taxonomic scrutiny has not been thorough, and species recognition is limited to a few traditional data sets, untested by additional studies and tools (Meyer & Paulay, 2005). The shallow-water benthic octopus is currently one such group, and further accumulation of DNA datasets associated with taxonomically well-examined voucher specimens as well as comprehensive taxonomic and phylogenetic frameworks is required for effective identification through DNA barcoding.

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